Ho Laboratory-Material and Methods

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (2015).

Sample Collection

At necropsy, the weight of the uterus was recorded prior to dividing the uterus longitudinally in two halves. The right half (one uterine horn) was fixed in 10% neutral buffered formalin (NBF) and transferred to 70% ethanol. The left half (one uterine horn) was cut transversally in three pieces; the 1/3 from the proximal end closest to the left ovary was flash-frozen in liquid nitrogen, while the remaining two pieces from the middle and distal portions were individually embedded in Optimal Cutting Temperature (OCT) compound and frozen on dry ice. The fixed and frozen uterine samples were shipped from NCTR to Ho laboratory. The samples were collected from animal set 4 [postnatal day (PND) 21], 6 (PND 90), 8 (6 months), and 10 (1 year) from all BPA and EE_2 dose groups, from the continuous dosing arm.

Whole-genome RNAseq data collection

Four flash-frozen samples from each group (control, BPA2.5, BPA25, BPA250, BPA2500, BPA25000 EE2-0.5 and EE2-0.05) were selected for RNAseq analyses. All uteri samples were collected from one-year-old rats and all were at estrous stage. Total RNA was extracted from uterine samples using a Qiagen RNeasy Lipid kit (Qiagen). The RNA quality and quantity were assessed using Agilent Bioanalyzer (Agilent) and NanoDrop ND-1000 spectrophotometer (Thermo Scientific), respectively. RNA libraries were prepared according to manufacturer's protocol of TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) and were sequenced with HiSeq1000 sequencing system in the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati. Sequence reads were aligned to the reference *Rattus norvegicus* genome (rn5) using the TopHat2 aligner (Trapnell et al., 2009) followed by quality control (Andrews, 2010; Deluca et al., 2012). Reads aligning to each known transcript were counted and all follow up analyses were performed using Bioconductor packages for next-generation sequencing data analysis (Huber et al., 2015). The differential gene expression analysis was performed based on the negative-binomial statistical model of read counts as implemented in the *edgeR* Bioconductor package (Anders et al., 2013) for each comparison separately. P-values were FDR-adjusted for multiple testing using the false discovery rates (Storey and Tibshirani, 2003) and gene expression profiles in the heatmap were clustered using Bayesian infinite mixture model (Freudenberg et al., 2010). The differential gene expression analysis was followed by enrichment analysis performed with CLEAN package (Freudenberg et al., 2009). The RNAseq data were collected with knowledge of treatment group.

Histology and Immunohistochemical Analyses:

Formalin-fixed samples were processed for hematoxylin and eosin (H&E) staining and scored for~~,~~ hyperplasia, squamous metaplasia and other endometrial changes like endometrial breakdown and

inflammatory response. All samples were scored by a certified pathologist, on the basis of representative H&E-stained sections. H&E staining was done for sections from 4 time points (PND 21, PND 90, 6 months, and 1 year). Immunohistochemical (IHC) analyses on proliferating cell nuclear antigen (PCNA) and ERalpha using specific antibodies (PCNA from Millipore; ERalpha from Santa Cruz) were performed according to our standard protocol (Zhu et al., 2004). Apoptotic cells were stained using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Millipore). The IHC signals were scored by a certified pathologist in a blinded manner.

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